

**Modified Forms of Hepatitis C NS3 Protease for Facilitating Inhibitor Screening
and Structural Studies of Protease:Inhibitor Complexes**

This application claims priority from provisional U.S. Application Serial No.
5 60/115,271, filed January 8, 1999, which is incorporated herein by reference in its
entirety.

Technical Field of the Invention

The present invention relates to modified forms of the Hepatitis C NS3
protease. The wild type protease is essential *in vivo* for viral replication of Hepatitis
10 C. The novel proteins of this invention are useful for screening for inhibitors of the
protease and for structural studies of the protease and protease:inhibitor complexes.

Background of the Invention

Hepatitis C virus (HCV) infection is the suspected cause of 90% of all cases of
15 non-A, non-B hepatitis (Choo et al., 1989, Kuo et al., 1989). HCV infection is more
common than HIV infection with an incidence rate of 2-15% worldwide. Over 4
million people are infected with HCV in the United States alone. While primary
infection with HCV is often asymptomatic, almost all HCV infections progress to a
chronic state that persists for decades. A staggering 20-50% are thought to eventually
20 develop chronic liver disease (e.g. cirrhosis) and 20-30% of these cases will lead to
liver failure or liver cancer. Up to 12,000 people in the U.S. will die this year from
sequelae associated with HCV infection. As the current population ages over the next
two decades, the morbidity and mortality associated with HCV are expected to triple.
The development of safe and effective treatment(s) for HCV infection is a major
25 unmet medical need.

The established principle for antiviral intervention is the direct inhibition of
essential, virally encoded enzymes. The only approved treatment for HCV infection
is interferon, however, which indirectly effects HCV infection by altering the host
immune response. Interferon treatment is largely ineffective, as a sustained antiviral
30 response is produced in less than 30% of treated patients. A safe and effective
antiviral treatment that blocks viral replication directly would likely have a much
more beneficial impact on the public health for HCV infection than does interferon
treatment. There have been no such inhibitors of HCV replication disclosed, to date.



23914

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Vaccination to prevent HCV disease has not shown promise due to the lack of efficacy of vaccine candidates for HCV.

Hepatitis C virus is a positive-strand RNA virus of the family *Flaviviridae*. The HCV genome encodes a single polyprotein of 3033 amino acids, of which
 5 residues 1026 to 1657 (631 amino acids) represent the NS3 protein (Choo et al., 1991). The HCV NS3 protein is a site-specific protease that cleaves the HCV polyprotein selectively at four sites related by their primary amino acid sequences (Grakoui et al., 1993a). These cleavages give rise to the mature non-structural (replicative) proteins of HCV, including NS3, NS4A, NS4B, NS5A, and NS5B
 10 (Bartenschlager et al., 1993; Grakoui et al., 1993b; Hijikata, et al., 1993a,b; Tomei et al., 1993; Bartenschlager et al., 1994; Eckart et al., 1994; Lin et al., 1994; Manabe, et al 1994). Genetic studies have demonstrated that the homologous NS3 proteases of related viruses (e.g. Yellow Fever Virus and Bovine viral diarrhea virus) are absolutely essential for viral replication (Chambers et al., 1990; Xu et al., 1997).
 15 Thus, inhibitors of NS3 protease should inhibit HCV replication and would be useful for the discovery and development of effective antiviral treatments for HCV infection.

Efficient processing of the HCV polyprotein by NS3 also requires the NS4A protein, amino acids 1658-1712 (58 amino acids) of the HCV polyprotein (Bartenschlager et al., 1994; Overton et al., 1994; Bartenschlager et al., 1995;
 20 Bouffard et al., 1995; Tanji et al., 1995). NS4A stimulates protease activity through the formation of a heteromeric complex with NS3 (Bartenschlager et al., 1995; Lin et al 1995; Satoh et al., 1995). NS4A is also thought to target the localization of the NS3 protease to the ER membrane, the likely site of viral replication (Hijikata et al., 1993b; Lin and Rice, 1995; Tanji et al., 1995). Studies to map the functional domains
 25 of NS3 and NS4A have demonstrated that the protease catalytic domain of NS3 resides within amino acids 1-181 (Bartenschlager et al., 1994; Tanji et al., 1994; Failla et al., 1995; Shoji et al., 1995) and that the catalytic domain interacts with, and is stimulated by, NS4A (Hijikata et al., 1993a; Lin et al., 1994; Bartenschlager et al., 1995; Failla et al., 1995; Satoh et al., 1995; Tanji et al., 1995). The remaining 450
 30 amino acids of NS3 comprise a functional domain with helicase and ATPase activities which are thought to be involved in viral genome replication (Jin and Peterson, 1995). Functional studies of NS4A *in vitro* demonstrated that the protease stimulatory

activity mapped to amino acids 21-34 of NS4A (Lin et al., 1995; Tomei et al., 1995; Shimizu et al., 1996). The N-terminal 20 amino acids of NS4A, on the other hand, are largely hydrophobic in nature and might serve as a transmembrane anchor domain (Lin and Rice, 1995).

- 5 The three-dimensional structure of the protease catalytic domain of NS3 has been determined by X-ray crystallography, with and without a cofactor peptide from NS4A (Kim et al., 1996; Love et al., 1996; Yan et al., 1998). These structures revealed very strong structural homology to chymotrypsin-like serine protease domains with the canonical catalytic triad comprising Ser-139, His-57, and Asp-81.
- 10 The N-terminal 28 amino acids of NS3 were unique, however, as they were unstructured in the absence of NS4A, while in the presence of NS4A peptide this region adopts β -strand and α -helix secondary structures. The co-crystal structure revealed that the NS4A peptide is inserted into, and partially buried by, adjacent β -strands of NS3. Local rearrangements near the protease active site also occur as a
- 15 result of NS4A binding, and these are thought to render the protease more catalytically active. Thus, NS4A would be expected to stabilize the active conformation of the HCV protease.

- Near the N-terminus of NS3 is an α -helix spanning residues 13-21 (α -helix 0) that appears to be stabilized by the NS4A peptide. The external face of this helix is
- 20 very hydrophobic and consists entirely of branched aliphatic residues. Due to its hydrophobic nature, it has been speculated that this surface might be involved in additional membrane interactions for anchoring the NS3:NS4A complex to cytoplasmic membranes (Yan et al., 1998).

- Routine methods for the expression of recombinant NS3 protease (e.g. *E. coli*,
- 25 baculovirus) have been employed widely. A common problem encountered when expressing wild-type NS3 protease (either full-length or truncated catalytic domain) has been the production of either insoluble or poorly soluble protein, especially when using *E. coli* vector systems. The best systems described to date have produced low levels of recombinant wild-type protease and the protease tends to be poorly soluble
- 30 (Shoji et al., 1995; Suzuki et al., 1995; Hong et al., 1996; Steinkuhler et al., 1996). As many of these preparations are enzymatically active, this approach has sufficed to generate active enzyme for activity analysis and inhibitor screening. However, to

carry out structural studies, highly expressed enzymes characterized by high solubility and low aggregation, in addition to enzymatic activity, are required.

Efforts have been made to overcome problems associated with low expression and/or poor solubility of the HCV protease, by constructing genetically engineered fusion derivatives of the native NS3 protease domain. Most notable are the generation of NS3 protease catalytic domains that form slowly-growing crystals suitable for structure determination by X-ray crystallography (Love et al., 1996; Kim et al., 1996; Yan et al., 1998). These have involved the construction of genetically engineered derivatives of NS3 by fusing polypeptide tags to the N-terminus and/or C-terminus that enhance the stable expression and/or solubility of the expressed protein (e.g. basic amino acids, poly-histidine). Other types of protease fusions (e.g. with ubiquitin, glutathione-S-transferase, maltose binding protein), including fusion of the NS4A protein to the C-terminus of the protease catalytic domain (Inoue et al., 1998), have been described that are partly soluble when expressed in *E. coli*, but few if any of these have overcome the critical limitation of low overall solubility. Very recently, bacterial expression of constructs in which the NS4a segment is fused to the N-terminus of the NS3 protease have been reported (Taremi et al., 1998; Pasquo et al., 1998); however, overall solubility of the final preparations were not reported.

There has been no published report of a NS3 preparation that is suitable for protein NMR work, as NMR studies typically require protein preparations that are expressed at high levels, are very highly soluble (>1 mM), and do not form soluble aggregates when purified. In addition, no X-ray structures of HCV protease complexed with enzyme inhibitors have been reported to date.

Summary of the Invention

At this time, no known pharmaceutical agent is available to prevent or cure HCV infection. HCV replication is dependent upon the activity of the virally encoded NS3 protease. Thus, elucidation of a specific inhibitor of this protease activity would be useful for the discovery of drugs to block HCV replication. This can be achieved using one or a combination of methods including, but not limited to: screening for small molecule inhibitors to serve as leads for medicinal chemistry; and the analysis of the three-dimensional structures (by X-ray crystallography or NMR) of complexes

between the HCV NS3 protease and compounds that bind to it in efforts to discover insights as to how the compounds might be chemically modified to produce potent inhibitors of this viral protease.

This invention enables the discovery of drugs that prevent or cure HCV infection. This invention encompasses novel, highly soluble, modified forms of HCV NS3 protease. More specifically, this invention includes novel, highly soluble, modified HCV NS3 proteases and novel, highly soluble, modified HCV NS3-NS4a fusion proteases. These novel proteins greatly facilitate screening for small molecule inhibitors and analysis of the three-dimensional structures, through X-ray crystallography and NMR spectroscopy, of complexes between the HCV NS3 protease and compounds that bind to it.

The present invention results from a number of significant modifications made to the wild-type HCV protease sequence.

One aspect of the invention is a modified HCV NS3 protease comprising an HCV NS3 protease comprising at least one substitution in the HCV NS3 protease of a hydrophobic α -helix 0 amino acid residue to a hydrophilic amino acid residue.

Another aspect of the invention is a modified HCV NS4a-NS3 fusion protease comprising a modified HCV NS3 protease fused to a HCV NS4a or modified HCV NS4a.

Further aspects of the invention are nucleic acid molecules encoding the proteins of the present invention, vectors and host cells.

A further aspect of the invention is methods of making proteins of the present invention.

Brief Description of the Figures

- Figure 1. Helical wheel representations of α -helix 0 (residues 13-21) of HCV protease. Residues in bold font are solvent exposed. (Top) Amino-acid sequence of wildtype α -helix 0; (Left) wild-type α -helix 0; (Right) Amino-acid substitutions in the α -helix 0 variants (X = His, Lys, Glu, Gln, Asp, or Asn) (see Experiments 3 and 4).
- Figure 2. Diagram of construction of modified HCV NS4a-NS3 fusion proteases. NS4a (residues 21-31) are fused to the N-terminus of NS3 in these diagrams by

way of a linker. “X” denotes a sequence change relative to SEQ ID NO:3. “N” and “C” denote the N- and C-termini of the constructs, respectively.

- Figure 3. Diagram of bacterial selection scheme for obtaining soluble HCV protease mutants. See Example 4 for a detailed description of the system.

(Center) expression plasmid (expressing HCV NS4a-NS3 fusion protease and modified Tet repressor) and chromosomally encoded Tet promoter-CAT (chloramphenicol acetyl transferase) gene fusion; (Right) case if NS3 protease is insoluble (activity masked by insolubility of the protease resulting in chloramphenicol-sensitive bacteria); (Left) case if NS3 protease is soluble (protease is active resulting in chloramphenicol-resistant bacteria).
- Figure 4. SDS-PAGE analysis of expression of various HCV NS4a-NS3 fusion protein constructs. Plasmid containing cells were grown to OD₆₀₀-0.7 and 10 ml cultures were induced with 0.25 mM IPTG for 20 hours at 20 degrees C. Cells were harvested by centrifugation (1500 rfc) in a tabletop microfuge and cell pellets were resuspended in 1ml of 25mM Na-phosphate buffer,pH 7.5; 0.5M NaCl, 2mM DTT, 10:M ZnCl, 10mM MgCl,10:g/ml DNase and sonicated twice for 1 min at power 5 in pulse mode. The homogenates were spun down in tabletop microfuge at max speed (20800 rfc) for 20min. Homogenates and supernatants were analyzed on 10-20% SDS-PAGE pre-cast gels (Bio-Rad). Lane 1, molecular weight standards. The following samples are in pairs of homogenate and supernatant, respectively: Lanes 2 & 3, parental fusion (SEQ ID NO:3); Lanes 4 & 5, helix0-1 mutations only (SEQ ID NO:12); Lanes 6 & 7, optimized linker only (SEQ ID NO:24); Lanes 8 & 9, helix0-1 mutations with optimized linker (SEQ ID NO:14).
- Figure 5. NMR analysis of modified HCV NS4a-NS3 fusion proteases +/- optimized linker. 2D ¹H-¹⁵N HSQC spectra were obtained for ¹⁵N-labeled mutant HCV NS4a-NS3 fusion proteases (all having the Helix0-1 sequence [see Figure 11 - SEQ ID NO:6] and purified as outlined in Example 7). Panel A – with non-optimized linker (see Example 4, SEQ ID NO:12); Panel B – with optimized linker (see Example 5, SEQ ID NO:14); Panel C –with optimized linker and

A40T, I72T, P86Q, C47S, C52L, C159S mutations (see Example 6, SEQ ID NO:18).

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 - Figure 6 shows an alignment of the amino acid sequences of SEQ ID Nos: 1, 3, 12, 14, 16, 18, 20, 22 and 24. Bolded letters with stippling indicates residue positions that are mutated relative to SEQ ID NO:1.
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 - Figure 7. Overlaid NMR ^1H - ^{15}N NQSQC spectra of a modified HCV NS4a-NS3 fusion protease with optimized linker (SEQ ID NO: 18) in apo-form and complexed with a peptide inhibitor (see Example 9). Apo-protease (thin grey line), peptide-complexed protease (thick black line).
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 - Figure 8. Portion of the electron density map of a modified HCV NS4a-NS3 fusion protease (SEQ ID NO:18) complexed with a peptide inhibitor (see Example 10). Two residues of the peptide inhibitor are shown: Cys₁ and Cha₂.
 - Figure 9. Amino acid sequence of (SEQ ID NO: 1) and nucleic acid sequence encoding (SEQ ID NO: 2) the parental non-fusion wild type HCV NS3 protease sequence.
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 - Figure 10. Amino acid sequence of (SEQ ID NO: 3) and nucleic acid sequence encoding (SEQ ID NO: 4) the initial HCV NS4a-NS3 fusion protease.
 - Figure 11. Amino acid sequence (SEQ ID NO: 5) of the α -helix 0 region of wild type HCV NS3 protease, and amino acid sequences of (SEQ ID NOS: 6-11) α -helix 0 regions (helix0-1, helix0-3, helix0-4, helix0-7, helix0-8, and helix0-10 respectively) of various soluble modified HCV NS4a-NS3 fusion proteases that are resistant to high levels of chloramphenicol in the bacterial selection scheme (see Example 4).
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 - Figure 12. Amino acid sequence of (SEQ ID NO: 12) and nucleic acid sequence encoding (SEQ ID NO: 13) a modified HCV NS4a-NS3 fusion protease with the α -helix 0 variant sequence helix0-1

- Figure 13. Amino acid sequence of (SEQ ID NO: 14) and nucleic acid sequence encoding (SEQ ID NO: 15) a modified HCV NS4a-NS3 fusion protease with the α -helix 0 variant sequence helix0-1 and an optimized linker sequence
- 5 • Figure 14. Amino acid sequence of (SEQ ID NO: 16) and nucleic acid sequence encoding (SEQ ID NO: 17) a modified HCV NS4a-NS3 fusion protease with the α -helix 0 variant sequence helix0-1, an optimized linker sequence, and surface mutations
- 10 • Figure 15. Amino acid sequence of (SEQ ID NO: 18) and nucleic acid sequence encoding (SEQ ID NO: 19) a modified HCV NS4a-NS3 fusion protease with the α -helix 0 variant sequence helix0-1, an optimized linker sequence, surface mutations, and cysteine mutations
- 15 • Figure 16. Amino acid sequence of (SEQ ID NO: 20) and nucleic acid sequence encoding (SEQ ID NO: 21) a modified HCV NS4a-NS3 fusion protease with the α -helix 0 variant sequence helix0-7, an optimized linker sequence, surface mutations, and cysteine mutations
- Figure 17. Amino acid sequence of (SEQ ID NO: 22) and nucleic acid sequence encoding (SEQ ID NO: 23) a modified HCV NS4a-NS3 fusion protease with the α -helix 0 variant sequence helix0-7, optimized linker sequence, surface mutations, cysteine mutations and C16T mutation
- 20 • Figure 18. Amino acid sequence of (SEQ ID NO: 24) and nucleic acid sequence encoding (SEQ ID NO: 25) a NS4a-NS3 fusion protein with wild-type α -helix 0 sequence and optimized linker sequence

Definitions

25 The following definitions are provided to more clearly delineate what is contemplated in this invention.

- “HCV” refers to the hepatitis C virus.

- “HCV NS3” refers to the protein fragment of the HCV polyprotein from any wild type strain of HCV that corresponds to residues 1026 - 1657 of the HCV polyprotein (as defined in Choo et al. *Proceedings of the National Academy of Sciences USA* **88**, 2451-2455 [1991]). The numbering convention for HCV NS3 throughout this application starts with residue 1 corresponding to residue 1026 of the HCV polyprotein, which is the first amino-acid residue of the mature processed NS3 protein fragment. HCV NS3 has portions which confer protease activity, helicase activity, and ATPase activity.
- “HCV NS3 protease” refers to any portion of the wild type HCV NS3 that has protease activity, not restricted to, but commonly associated with, HCV NS3 protease domain; or any wild type peptide that exhibits the protease activity associated with HCV NS3.
- “HCV NS3 protease domain” refers to the portion of wild type HCV NS3 that confers protease activity, usually encompassing HCV NS3 residues 1-181, but sometimes differing by the inclusion or deletion of residues at either the N- or C-terminus.
- “Modified HCV NS3 protease” refers to a peptide or protein whose sequence is an alteration from a wild-type HCV NS3 protease sequence and that exhibits the protease activity of HCV NS3 protease. Such modifications include, but are not limited to, naturally-occurring amino acid substitutions, non-naturally-occurring amino acid substitutions, conservative amino acid substitutions, amino acid insertions, amino acid deletions, and amino acid additions. Non-sequence modifications, including changes in acetylation, methylation, phosphorylation, carboxylation, or glycosylation, are also included in the definition of modified.
- “HCV NS4a” refers to the protease-stimulating protein fragment of the HCV polyprotein from any wild type strain of HCV that corresponds to residues 1658-1712 of the HCV polyprotein (as defined in Choo et al. *Proceedings of the National Academy of Sciences USA* **88**, 2451-2455 [1991]), any fragment thereof that exhibits protease-stimulating activity, or any wild type peptide that exhibits the protease-stimulating activity associated with residues 1658-1712 of the HCV

polyprotein. Full-length HCV NS4a particularly refers to residues 1-58, which correspond to residues 1658-1712 of the polyprotein. The numbering convention throughout this invention for HCV NS4a starts with residue 1 corresponding to residue 1658 of the HCV polyprotein (same as the first residue of the mature processed HCV NS4a fragment).

- “Modified HCV NS4a” refers to a peptide or protein whose sequence is an alteration from a wild-type HCV NS4a sequence and that exhibits the protease-stimulating activity of HCV NS4a. Such modifications include, but are not limited to, naturally-occurring amino acid substitutions, non-naturally-occurring amino acid substitutions, conservative amino acid substitutions, amino acid insertions, amino acid deletions, and amino acid additions. Non-sequence modifications, including changes in acetylation, methylation, phosphorylation, carboxylation, or glycosylation, are also included in the definition of modified.
- “Modified HCV NS4a-NS3 fusion protease” refers to a modified HCV NS3 protease fused to a HCV NS4a or modified HCV NS4a. A modified HCV NS4a-NS3 fusion protease may include an optimized linker sequence.
- “Modified forms of HCV NS3 protease” refers to the totality of the invention described herein and encompasses modified HCV NS3 proteases, modified HCV NS4a-NS3 fusion proteases, or both.
- “Naturally-occurring amino acid” refers to any of the 20 standard L-acids that occur in a referred-to position in any wild type HCV NS3 protease or wild type HCV NS4a.
- “Non-naturally-occurring amino acid” refers to any of the 20 standard L-amino acids that do not occur in a referred-to position in any wild type HCV NS3 protease or wild type HCV NS4a, D-amino acids, and synthetic amino acids such as β or γ amino acids.
- “Conservative amino acid substitution” refers to the substitution of one amino acid for another with similar characteristics, e.g., substitutions within the following groups: valine and glycine; glycine and alanine; valine and isoleucine

and leucine; aspartic acid and glutamic acid; asparagine and glutamine; serine and threonine; lysine and arginine; and phenylalanine and tyrosine. Other conservative amino acid substitutions can be taken from the table below.

Table 1

5

Conservative amino acid replacements

For Amino Acid	Code	Replace with any of:
Alanine	A	D-Ala, Gly, beta-Ala, L-Cys, D-Cys
Arginine	R	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-Met, D-Ile, Orn, D-Orn
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala, Pro, D-Pro, β -Ala, Acp
Isoleucine	I	D-Ile, Val, D-Val, Leu, D-Leu, Met, D-Met
Leucine	L	D-Leu, Val, D-Val, Met, D-Met, Ile, D-Ile,
Lysine	K	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val
Phenylalanine	F	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, cis-3,4, or 5-phenylproline
Proline	P	D-Pro, L-1-thioazolidine-4-carboxylic acid, D- or L-1-oxazolidine-4-carboxylic acid
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Tyrosine	Y	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Valine	V	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met

- “Hydrophobic amino acid” refers to amino acid residues whose sidechains are relatively non-polar, including, but not limited to, alanine, phenylalanine, isoleucine, leucine, methionine, proline, valine, and tryptophan.
- 5 • “Hydrophilic amino acid” refers to amino acid residues whose sidechains are relatively polar, including, but not limited to, aspartate, glutamate, lysine, asparagine, glutamine, arginine, serine, threonine, histidine and tyrosine.
- “ α -helix 0” refers to the sequence consisting of HCV NS3 residues Leu₁₃ through Leu₂₁ that takes on an alpha-helical structure when HCV NS3 protease is
10 complexed with a HCV NS4a segment (as in Kim et al, *Cell* **87**, 343-355 [1996] or Yan et al., *Protein Science* **7**, 837-847 [1998]).
- “Linker” refers to, in a modified NS4a-NS3 or NS3-NS4a fusion protease, a polypeptide sequence that joins the HCV NS4a sequence with the HCV NS3 sequence.
- 15 • “Optimized linker” refers to, in a modified NS4a-NS3 fusion protease, a linker sequence that joins the NS4a and NS3 sequences such that the resulting fusion protein has enhanced stability and solubility characteristics relative to a non-optimized linker.
- “Zinc-binding cysteine residues” refer to the naturally-occurring cysteine residues
20 Cys₉₇, Cys₉₉, and Cys₁₄₅ in HCV NS3.
- “Non-zinc-binding cysteine residues” refer to the naturally-occurring cysteine residues Cys₄₇, Cys₅₂, and Cys₁₅₉ in HCV NS3.

Detailed Description of the Invention

- 25 The benefit of this invention is that the modified forms of HCV NS3 protease retain full activity, yet are highly amenable to biochemical experimentation because of their highly soluble (> 30 mg/ml) and non-aggregating nature under detergent-free conditions. In contrast, wild-type forms of the HCV NS3 protease (domain) require

detergents for solubilization. Because the modified forms of HCV NS3 protease of the invention exhibit very high degrees of solubility in the absence of detergents, they are well suited for NMR and X-ray crystallographic structure determination of HCV NS3 protease complexed with inhibitors, facilitating iterative structure-based drug design efforts with this pharmacologically important enzyme. Their solubility without the use of detergents also makes them very useful in screening assays for inhibitors.

As previously noted, one aspect of the invention is a modified HCV NS3 protease comprising at least one substitution in HCV NS3 protease of a hydrophobic α -helix 0 amino acid residue to a hydrophilic amino acid residue.

Another aspect of the invention is a modified HCV NS4a-NS3 fusion protease comprising a modified HCV NS3 protease fused to a HCV NS4a or modified HCV NS4a.

Various combinations in which hydrophobic amino acids are substituted to hydrophilic amino acids in the α -helix 0 are described elsewhere in the specification and in the claims. In a preferred embodiment, the hydrophobic α -helix 0 amino acid residues are selected from the group consisting of Leu₁₃, Leu₁₄, Ile₁₇, Ile₁₈, and Leu₂₁. In a more preferred embodiment, Leu₁₃ is substituted to glutamic acid, Leu₁₄ is substituted to glutamic acid, Ile₁₇ is substituted to glutamine, Ile₁₈ is substituted to glutamic acid, and Leu₂₁ is substituted to glutamine. (This is helix 0-1 in Figure 11, SEQ ID NO: 6.) In another more preferred embodiment, Leu₁₃ is substituted to glutamic acid, Leu₁₄ is substituted to glutamine, Ile₁₇ is substituted to glutamine, Ile₁₈ is substituted to lysine, and Leu₂₁ is substituted to histidine. (This is helix 0-7 in Figure 11, SEQ ID NO: 9.)

In another preferred embodiment of the invention, the modified HCV NS3 protease further comprises at least one substitution of a hydrophobic amino acid residue not in the α -helix 0 to a hydrophilic amino acid residue.

In an additional preferred embodiment, the modified HCV NS3 protease further comprises at least one substitution of a non-zinc-binding cysteine residue to a non-cysteine amino acid residue.

In a preferred embodiment, the HCV NS3 protease that is altered comprises approximately residues 1-181 of HCV NS3.

In a preferred embodiment of an aspect of the invention that is a modified HCV NS4a-NS3 fusion protease, the HCV NS4a that is altered or unaltered comprises approximately residues 21-31 of full-length HCV NS4a.

In an aspect of the invention that is a modified HCV NS4a-NS3 fusion protease, a preferred embodiment further comprises a linker comprising an optimized linker sequence. In a more preferred embodiment, the NS4a is linked to the amino terminus of the NS3. In a most preferred embodiment, the optimized turn sequence is Ser-Gly-Asp-Thr where Ser corresponds to NS4a residue Ser₃₂ and Thr corresponds to NS3 residue Thr₄.

In a preferred embodiment of an aspect of the invention that is a modified HCV NS4a-NS3 fusion protease, the modified HCV NS4a further comprises at least one substitution of a hydrophobic amino acid residue to a hydrophilic amino acid residue. In a most preferred embodiment, residue 30 is substituted to asparagine.

The invention also includes isolated nucleic acid molecules encoding the proteins of the present invention, vectors comprising said nucleic acid molecule, and host cells comprising said vectors. *E. coli* cells harboring plasmids containing certain nucleic acid molecules of the present invention were deposited with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA, 20110 USA, and have ATCC accession numbers 204070 and 204071.

The invention also includes methods of making proteins of the present invention using host cells of the present invention.

The present invention exhibits distinct differences from and improvements over the prior art. In contrast to the published works documenting attempts to solubilize the HCV NS3 protease by relying solely on the fusion of solubilizing tags or protein fusion partners to the protease (i.e Kim et al. 1996; Yan et al., 1998; Taremi et al., 1998), the present invention changes amino-acid residues *within* the HCV NS3 protease coding region itself, resulting in what is referred to herein as modified forms of the HCV NS3 protease. These modified forms retain full enzymatic activity.

The present invention provides solubility of greater than 30 mg/ml -- the highest reported level of detergent-free solubility for an HCV NS3 protease of any kind (wild type or engineered in any way).

Applicants have used the present invention to collect high quality NMR spectra. Presented herein are high quality NMR spectra of a modified form of HCV NS3 protease and of a modified form of HCV NS3 protease:inhibitor complex. These are the first reported instances of high quality NMR spectra of an HCV protease (wild type or engineered in any way) alone or in complex with an inhibitor.

Applicants herein present a modified form of HCV NS3 protease:inhibitor complex determined by X-ray crystallography and demonstrate that the proteins of the present invention can rapidly produce high quality co-crystals with protease inhibitors. This is the first reported instance of X-ray crystallography showing an HCV protease (wild type or engineered in any way) complexed with an inhibitor. The proteins of this invention are especially useful because of their ability to produce high quality co-crystals with protease inhibitors. Structure-based drug design with a protein is often limited by it's ability to form diffraction-grade co-crystals with inhibitors in a timely manner. A protein, such as that of the present invention, that can be co-crystallized quickly facilitates the iterative process of structure-based design work.

The modified forms of HCV NS3 protease of the present invention are also useful for screening for small molecules inhibitors of HCV NS3. Proteins of the present invention can be prepared in the absence of detergents, allowing for the identification of compound inhibitors that would otherwise be undetectable if screened in the presence of detergents. The modified HCV NS3 protease in the non-fusion form can also be used to study whether a compound interferes with the binding of NS4a to NS3.

The general strategy used to obtain these soluble modified HCV NS3 protease variants was to sequentially target key regions of the protein that might be important for protein solubility, mutagenize these targeted regions in a semi-random manner, and either select or screen for bacterial clones expressing protein variants that exhibited higher degrees of solubility. The steps used to make some of the preferred embodiments of the invention are outlined in the Examples. Over the course of the Examples, a progressively higher degree of amino-acid residue substitution (relative to the starting wild-type sequence) was generated until the production of modified forms of HCV NS3 protease that exhibited high levels of solubility with low levels of

aggregation was achieved. Many different hydrophobic-to-hydrophilic HCV NS3 protease surface residue substitutions (naturally-occurring and non-naturally-occurring) were combined with different NS4a-NS3 fusion linker sequences, and these protease mutants were either selected or screened to find modified proteins that exhibited the desired solubility characteristics. Some completely conserved hydrophobic residue positions in the α -helix 0 were targeted for mutagenesis because they made up a particularly extensive hydrophobic patch on the surface of HCV NS3 protease. Other HCV NS3 protease hydrophobic surface residues were also mutagenized; particularly good candidates were residues whose position was variable among the HCV NS3 sequences from other wild type HCV isolates. These and other substitutions are documented and included in the claims.

The following paragraphs describe in greater detail the invention claimed and ways of making it. While the order of the paragraphs follows the experimental process used, one of skill in the art can figure out ways to make the invention claimed in which the steps are done in different orders and/or steps are omitted.

Any HCV NS3 protease and HCV NS4a sequences can be used as a starting point for the modifications. Here, a cloned HCV isolate sequence was used as a starting point for the mutagenesis experiments (Example 1, Figure 9, SEQ ID NO: 2]. The expression system used features a synthetic gene in which all codons have been optimized for high-level expression in *E. coli*. While this may explain the high levels of expression observed for the resulting constructs, it is not essential to the invention and any nucleotide sequence encoding an HCV NS3 protease (using the standard genetic code) could be used to express these proteins. HCV NS3 protease includes any fragment of wild type HCV NS3 that exhibits protease activity or any wild type peptide that exhibits the protease activity associated with wild type HCV NS3 (as defined in the Definitions section). It is also not essential that the modified forms of HCV NS3 protease described here be expressed in *E.coli*. Any *in vivo* expression host (bacterial, insect, plant, mammalian, other) could be used to express these modified forms of HCV NS3 protease. Also, *in-vitro* production of these variants is possible. The present invention includes modified forms of HCV NS3 protease produced by any means.

The invention includes the use of HCV NS4a. See the Definitions section for the definition of HCV NS4a. In Example 2, the NS4a sequence comprising residues 21-31 (G₂₁ S₂₂ V₂₃ V₂₄ I₂₅ V₂₆ G₂₇ R₂₈ I₂₉ V₃₀ L₃₁) of full-length NS4a (SEQ ID NO: 26) was fused to the N-terminus of the HCV NS3 protease. The linker sequence in
5 this experiment was the simple dipeptide sequence asparagine-glycine. A variety of other linkers could be used, and one of ordinary skill in the art would be able to choose other appropriate linkers. The NS4a could also be fused to the C-terminus of the NS3. As has been demonstrated (Lin et al., 1995; Tomei et al., 1995; Shimizu et al., 1996), a NS4a peptide including residues 21-31 increases the activity of the HCV
10 NS3 protease *in vitro*. The linker described in Example 2 was initially used. However, better results (in terms of expression and solubility) were obtained from the optimized linker constructs resulting from the experiments described in Example 5. While others have very recently published other NS4a-NS3 linkers (Taremi et al., 1998; Pasquo et al., 1998), the optimized linkers documented in this invention (in
15 combination with the other mutations described here) confer unprecedented levels of protease solubility.

The first α -helix of HCV NS3, known as α -helix 0, has an extremely hydrophobic solvent-exposed surface (Yan et al, 1998), and applicants believed that this could be a contributor to the insoluble character of preparations of wild type HCV
20 NS3 protease (such as SEQ ID NO: 1 in Figure 9) and unmodified HCV NS4a-NS3 fusion protein (such as SEQ ID NO: 3 in Figure 10). Therefore, the targeted/semi-random mutagenesis method was applied in an effort to change the hydrophobic solvent-exposed residues of α -helix 0 to more hydrophilic residue types (see Examples 3 and 4). Currently, all known strains of HCV have five hydrophobic
25 solvent-exposed residues in α -helix 0 (L₁₃, L₁₄, I₁₇, I₁₈, L₂₁), and, according to this invention, these could be changed alone or in any combination. Applicants chose to mutate all five in tandem. Changing other solvent-exposed hydrophobic residues in α -helix 0 in strains currently unknown is also encompassed by this invention, as is changing hydrophobic residues in α -helix 0 that are not solvent-exposed. Although
30 applicants demonstrate changes to the α -helix 0 of a HCV NS4a-NS3 fusion protease, the unfused HCV NS3 protease could also undergo such changes and a such modified HCV NS3 protease is encompassed by this invention.

A simple method of isolating a soluble modified form of HCV NS3 protease from a large library of candidate mutants is to simply screen for the presence of modified HCV NS4a-NS3 fusion protein variants in the cleared supernatants of induced transformants by SDS-PAGE analysis, similar to the analysis depicted in Figure 4. This method is laborious and inherently low-throughput. Another, much more powerful high-throughput method is to have a system where the most soluble modified forms of HCV NS3 protease are selected from among all the other clones in the library. Applicants have used such a selection scheme to select for more soluble modified forms of HCV NS3 protease. (See Example 4 and Figure 3.) What applicants describe is a specific application of a general method for selecting for soluble (or active) variants of proteases. This scheme can be used to screen for solubility or activity of any of the modified forms of HCV NS3 protease encompassed by this invention.

The modified HCV NS4a-NS3 fusion proteases having α -helix 0 mutations only (described in Example 4) retained full enzymatic activity and were considerably more soluble than the non-mutated NS4a-NS3 fusion (see Figure 4, compare lanes 3 and 5). These mutants are useful for experiments where the protein concentration can be kept relatively low, such as enzyme assays. Also, these mutants allow the bacterial selection system (Figure 3) to be used as a screening system for HCV protease inhibitors. (Inhibitors will cause a decrease in the growth of these induced bacteria in chloramphenicol media.) Similarly, the modified HCV NS3 proteases that are not fusions could be used in enzyme assays and to screen for inhibitors. However, the modified HCV NS4a-NS3 fusion proteases having only α -helix 0 mutations still had a tendency to aggregate at the high protein concentrations required for protein NMR (see Figure 5, panel A). One concern was that the NS4a and NS3 segments were not fused optimally. Therefore, work was done to optimize the linker sequence connecting the NS4a and NS3 segments.

Many different methods can be used to optimize a linker sequence. In general, the goal is to successfully connect two protein segments using a polypeptide linker so that the relative spatial positioning of the two segments is maintained with minimal stress and perturbation to the structure. Another important aspect is that the linker should ideally not be highly flexible, as this might tend to destabilize the protein. In

this case, because the desired relative structural positioning of the NS4a and NS3 segments was known, a structure-based approach was taken to find an optimal linking sequence. As described in Example 5, the structural information in the Brookhaven Protein Data Base (PDB) was mined to find short turn sequences that successfully

5 linked two beta-strands that were structurally similar to the NS4a (residues) and NS3 (residues) segments as seen in the published X-ray structure of the HCV NS3 protease / NS4a peptide complex. Coordinates from Protein Database file 1JXP.pdb (Yan et al., *Protein Science* 7, 837-847 [1998]) were used for this purpose. In addition, the sequential proximity of a surface-exposed hydrophobic residue within the NS4a

10 segment (Val₃₀) provided the opportunity to test the effect of mutation of this residue while trying the different linker sequences.

One optimal linker sequence among those tested conferred high levels of expression and solubility. This optimized linker sequence in combination with the wild-type α -helix 0 (SEQ ID NO: 24 in Figure 18) does not confer solubility to the

15 protease while the optimized turn in combination with a modified α -helix 0 (SEQ ID NO: 14 in Figure 13) does (Figure 4, compare lanes 7 & 9). Overall, the quality of the NMR spectra obtained with the modified α -helix 0 / optimized linker form of the protease (SEQ ID NO: 14 - Figure 5, panel B) was superior to that obtained with the modified α -helix 0 / non-optimized linker form of the protease (SEQ ID NO: 12 -

20 Figure 5, panel A), indicating a more soluble and less-aggregating form of the protease.

The effect of additional solvent-exposed hydrophobic-to-hydrophilic amino-acid substitutions was explored by changing certain surface residues in SEQ ID NO:14, resulting in SEQ ID NO:16 (see Example 6). The residues were chosen by

25 aligning HCV NS3 protease sequences from published sequences of wild type HCV isolates and looking for naturally-occurring hydrophobic-to-hydrophilic amino-acid substitutions at residue positions that are solvent exposed in published NS3 protease structures. The combination of three of these naturally-occurring substitutions (A40T, I72T, P86Q) were found to have a solubility enhancing effect (SEQ ID NO: 16 in

30 Figure 14). In addition, three of the four non-zinc binding cysteine residues were targeted for amino-acid substitutions (SEQ ID NO: 18 in Figure 15). Other sequences generated by this process can also yield useful results. Two variants of the Helix0-7

sequence variant (see Figure 11 - SEQ ID NO. 9) was substituted for the Helix0-1 sequence (see Figure 11 - SEQ ID NO. 6) to generate SEQ ID NOS: 20 and 22. These modified HCV NS4a-NS3 fusion protease variants were also highly soluble.

In order to demonstrate the usefulness of the resulting protease variants for structural studies of the protease and protease:inhibitor complexes, the modified HCV NS3 protease encoded by SEQ ID NO: 18 was characterized by multi-dimensional NMR spectroscopy (Examples 8 and 9) and X-ray crystallography (Example 10).

In Example 8, NMR spectroscopy of the apo-form of the protease was used to generate sequential backbone assignments and sidechain NMR assignments. These assignments include the catalytic triad residues His₅₇, Asp₈₁, and Ser₁₃₉ and the residues spatially near to them, indicating that spectra of the apo-form of the protein is a useful tool for analyzing of the effect of addition of potential NS3 protease inhibitors using chemical shift perturbation mapping. As shown in Example 9, addition of a published peptidic HCV protease inhibitor causes many chemical shift perturbations for residues in the ¹H-¹⁵N HSQC NMR spectrum of the protease, including the active site residues. This is the first publication of high quality NMR spectra of HCV NS3 protease and of a HCV NS3 protease:inhibitor complex. As demonstrated here, the modified protease yields high quality NMR spectra that can be used to identify compounds that bind to the protease. One can use this chemical-shift mapping technique to identify novel compounds that bind to the protease by collecting a series of spectra in which different compounds have been mixed with the protease.

Example 10 demonstrates that the modified HCV NS3 protease encoded by SEQ ID NO: 18 can be co-crystallized with a published HCV protease inhibitor overnight, resulting in high quality crystals that diffract to 2 Å. This is the first publication of a HCV NS3 protease:inhibitor complex solved by X-ray crystallography and demonstrates that proteins produced by application of this invention can rapidly produce high quality co-crystals with protease inhibitors. In addition, the high resolution structure verifies that the NS4a-NS3 fusion construct produces the relative structural positioning of the NS4a-NS3 polypeptide segments as designed.

The nucleic acid molecules of the present invention can be made by one of ordinary skill in the art using standard knowledge of codon usage and molecular biology techniques that can be found in, for example, "Molecular Cloning, A Laboratory Manual" (2nd edition, Sambrook, Fritch and Maniatis 1989, Cold Spring Harbor Press).

The vectors of the present invention which comprise nucleic acids of the present invention can be made using any suitable vector as determined by one of ordinary skill in the art. Such vectors include, but are not limited to, vectors such as pBR322 and expression vectors such as pET series (Novagen). The vectors of the present invention can be produced using standard molecular biology techniques as found in, for example, "Molecular Cloning, A Laboratory Manual" (2nd edition, Sambrook, Fritch and Maniatis 1989, Cold Spring Harbor Press).

Any suitable host cell can be used, such as bacterium, insect, plant, mammal or other. Conditions for expression and recovery of the proteins can be determined by one of ordinary skill in the art using techniques found in, for example, Protein Expression in Mammalian and Insect Cell Systems, S. Geisse and H.P. Kocher in Methods in Enzymology, Vol. 306 (1999), p. 19-42.

All references cited in this specification are incorporated herein by reference.

Examples

The following Examples explain how to make and use certain embodiments of the invention. From these Examples, the Detailed Description of the Invention, and the references cited therein, one of ordinary skill in the art can readily discern how to make these and other embodiments of the invention. The Examples are not meant to limit the scope of the invention; the scope of the invention is delineated by the claims.

In the following Examples, the standard residue numbering for HCV NS3 protease is used (as outlined in the Definitions). In cases where sequences are added to the N-terminus, the NS3 numbering remains the same, sometimes resulting in negative numbering for the additional N-terminal residues. All presented protein sequences are aligned in Figure 6.

Example 1 – Parental HCV protease DNA sequence

The HCV NS3-encoding DNA used as a basis for all the subsequent modifications is a synthetic gene coding for the HCV protease (residues 1-181) shown in SEQ ID NO: 2 (Figure 9). Residues 1-181 comprise the portion of the HCV NS3 gene product that exhibits protease activity. Longer fragments of the HCV NS3 protein could be used. The synthetic gene was constructed so that all codons were optimized for high level expression in *E.coli*. The protein-coding sequence of this construct is shown in SEQ ID NO:1 (Figure 9). This HCV protease protein is produced at a high level when expressed from vector pET24a (Novagen) in *E.coli* strain BL21(DE3) (Novagen), but upon fractionation of the extract the protease is in the insoluble fraction (data not shown).

Example 2 - Fusion of wild type NS4a to parental NS3 with a linker

A plasmid was constructed that encoded the following portion of the full-length HCV NS4a sequence: NS4a residues 21-31; G₂₁ S₂₂ V₂₃ V₂₄ I₂₅ V₂₆ G₂₇ R₂₈ I₂₉ V₃₀ L₃₁ (SEQ ID NO: 26). This portion of the NS4a sequence was fused to the amino-terminus of the HCV NS3 protease sequence (NS3 HCV protease sequence 5-183). The fusion was constructed so that the NS4a segment was fused to the NS3 segment by means of a linker (Asn Gly, aka NG), yielding the protein sequence ...GSVVIVGRIVLNGAYAQQ ... at the NS4a-NS3 fusion (see Seq ID NO:3 in Figure 10).

The expression plasmid for the NS4a-linker-NS3 fusion protease was constructed by a three-way ligation of the following three DNA preparations:

- 1) The vector for the expression plasmid was a modified form of pET28a (Novagen), where pET28a plasmid DNA had been double-digested with XhoI and SalI, and subsequently ligated, destroying both sites in the vector. The resulting modified vector (mpET28a) was double digested with NdeI and EcoRI.
- 2) Two synthetic 5'-phosphorylated oligonucleotides (coding for the NS4a and linker segments) were annealed, creating NdeI and XhoI sticky ends.

5'-
TATGAAAAAAAAAAGGATCCGTTGTTATCGTCGGCCGTATAGTACTGAACGGTGCTTACGC
TCGCAGAC-3'

5'-

TCGAGTCTGCTGAGCGTAAGCACCGTTCAGTACTATACGGCCGACGATAACAACGGATCC
TTTTTTTTTCA-3'

- 5 3) The NS3-coding DNA from Seq ID NO:1 was PCR amplified with the following oligonucleotides which created a silent mutation encoding a XhoI site. The resulting PCR fragment was digested with XhoI and EcoRI.

5'-CAGCAGACTCGAGGTCTGC-3'

10

5'-GCACGAATTCACGGGGAACGCATGG-3'

15 The plasmid product of this three-way ligation codes for a NS4a-NS3 fusion protein (see Seq ID NO:3 in Figure 10; SEQ ID NO: 3). This fusion protein is produced at a high level when expressed in *E.coli*, but upon fractionation of the extract the fusion protein is in the insoluble fraction (see Figure 4, lanes 2 & 3).

20 **Example 3- Generation of a large library of modified HCV NS4a-NS3 fusion proteases in which the hydrophobic solvent-exposed residues of α -helix 0 are replaced with hydrophilic residues**

The HCV NS3 protease sequence generated in Example 2 (Seq ID NO:3) was PCR amplified and moved into another expression vector as a NcoI-SalI fragment. This modified expression vector (depicted in Figure 3) is derived from pET21 (Novagen) and includes a modified Tet repressor in which a HCV NS3 protease site has been inserted. The vector was cut with NcoI and XhoI, and ligation with the NcoI-SalI fragment resulted in the destruction of the XhoI site within the pET21-derived multiple cloning site in this plasmid vector.

30 The five hydrophobic solvent-exposed residues in α -helix 0 (L₁₃, L₁₄, I₁₇, I₁₈, L₂₁) were singled out for targeted semi-random mutagenesis using the biased codon method (Kamtekar et al., *Science* **262**, p1680-1685 [1993]). In this method, a "VAV" codon (V = G, C, or A) encodes a mixture of six possible codons all coding for hydrophilic residue types (His, Glu, Gln, Asp, Asn, or Lys).

The targeted semi-random mutagenesis was carried out by using this oligonucleotide sequence as the 5' primer for PCR amplification of the HCV NS3 protease sequence:

5 XhoI
 Gln Thr Arg Gly *** ** Gly Cys *** ** Thr Ser *** Thr Gly Arg Asp
 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
CAG ACT CGA GGT VAV VAV GGT TGC VAV VAV ACC TCC VAV ACC GGT CGT GAC

10 where V = (G or C or A)

A 3' primer was used to prime at a site downstream of the NS3 protease stop codon and EcoRI site. The PCR products were digested with XhoI and EcoRI and ligated into the XhoI-EcoRI cleaved HCV NS4a-NS3 fusion protease expression
 15 vector described at the beginning of this Example.

In this way, a library of 7776 (= 6⁵) potential unique modified HCV NS4a-NS3 fusion proteases with different mutant hydrophilic α -helix 0 sequences was generated. Portions of the ligation mixture were electroporated into *E.coli* strain MC1061 (ATCC 5338). Over 50,000 transformants were pooled and plasmid DNA
 20 was isolated.

The plasmid used as a vector for the construction of this library is diagrammed in Figure 3 and is relevant to the mutant selection experiments described in Example 4. When another selection or screening method is used (such as screening by SDS PAGE analysis of homogenates of induced transformants [as in Figure 4]), other
 25 plasmid vectors would be suitable.

Example 4 – **Bacterial Selection of Soluble modified HCV NS4a-NS3 Fusion Proteases**

The bacterial selection system is diagrammed in Figure 3. The system features a
 30 plasmid encoding a mutagenized HCV protease gene (in this case, the modified HCV NS4a-NS3 fusion proteases with the α -helix 0 mutations) as well as a gene encoding a modified Tet repressor. The modification of the Tet repressor is the introduction of a HCV NS3 protease cleavage site within a solvent-exposed loop of the Tet repressor protein. The strain carrying this plasmid also has a chromosomally-encoded

chloramphenicol acetylase transferase gene (CAT - conferring chloramphenicol resistance) under the control of the Tet promoter. After induction of the modified HCV NS4a-NS3 fusion protease by IPTG (under lac-T7 control), the strain becomes either chloramphenicol resistant (Cm^R) if the protease activity is present (cleavage of the modified Tet repressor allowing expression of CAT) or remains chloramphenicol sensitive (Cm^S) if the fusion protease activity is not present (no cleavage of the modified Tet repressor and therefore there is repression of CAT).

In this case, the expressed wildtype HCV protease is inherently active, however the activity is masked by the insoluble character of the expressed protease, resulting in a Cm^S phenotype. If among the pool of mutagenized transformants a more soluble active mutant protease is expressed, the inherent activity of the protease is unmasked by the soluble nature of the mutant enzyme and the cells harboring this mutant protease become Cm^R.

Expression of the parental HCV NS4a-NS3 fusion protease (SEQ ID NO: 3) in this section system system yielded *E.coli* cells that grew only very slowly on agar plates with 1µg/ml chloramphenicol. The library of α-helix 0 mutants (described in Example 3) was transformed into the *E.coli* selection strain. Many plasmids encoding α-helix 0 mutagenized modified HCV NS4a-NS3 fusion proteases conferred upon induced transformed *E.coli* cells an enhanced ability to grow on plates with low levels of chloramphenicol (1-3 µg/ml chloramphenicol). However, twelve transformants grew on plates with very high levels of chloramphenicol (30 µg/ml). These highly Cm^R transformants were colony purified and solubilities of the expressed mutant HCV NS4a-NS3 fusion proteases were evaluated by SDS-PAGE analysis (similar to that depicted in Figure 4). Six of the transformants exhibited more soluble proteases than the others and plasmid DNA was prepared and sequenced. The relevant portions (through the NS3 α-helix 0 segment) of the sequences from these isolates are listed as SEQ ID Nos: 6-11 (see Figure 11).

As shown in Figure 4 (lanes 4 & 5), expression of a modified HCV NS4a-NS3 fusion protease with the Helix0-1 sequence (see SEQ ID NO: 6 and SEQ ID NO: 12) produced a protein that was in the soluble fraction while fusion protein with the wild-type α-helix 0 sequence was insoluble (lanes 2 & 3). Similar enhancements in solubility were obtained with the other five sequenced variants.

E. coli cells containing a similar expression system have been deposited with the ATCC and have ATCC accession number 207047. The cells submitted to the ATCC differ from the cells used here in that the ATCC cells have a plasmid containing a CMV protease and the CMV protease cleavage site within the Tet repressor, and in that the CAT gene is on a second plasmid, rather than on the chromosome. One of ordinary skill in the art could make a cells useful for the present invention from the cells deposited with the ATCC by replacement of the CMV protease sequences with HCV protease sequences and changing the CMV cleavage site coded within the Tet repressor gene to a HCV protease cleavage site. Cells useful for the present invention could have the CAT gene on a second compatible plasmid rather than on the chromosome.

The cells having ATCC accession number 207047 and the bacterial selection system referred to herein are further described in application U.S. Serial No. 60/115,270, filed on January 8, 1999, and application U.S. Serial No. _____, filed on even date herewith, both of which are incorporated herein by reference.

Example 5 –Linker optimization in a modified HCV NS4a-NS3 fusion protease, including change in NS4a

Structural information from the Protein Data Bank was used to identify structurally characterized proteins that have two β -strands (structurally homologous to residues NS4a residues G₂₇-R₂₈-I₂₉-V₃₀-L₃₁ and NS3 residues A₅-Y₆-A₇-Q₈-Q₉) linked by a tight turn (Searchloop function in the Insight II program, Molecular Simulations Inc.). Three different turn types were identified (as exemplified by the Brookhaven Protein DataBase (PDB) files 1OPB [residues 45-48], 1LID residues 109-112], and 1EUR [residues 177-184]) and three sets of degenerate double stranded oligonucleotides were synthesized that coded for 8 to 12 variants of each of the three turn types.

Turn oligo #1:

	Asn	Asn	Ser
GlyArgIleIleLeuSerGlyAspThrAlaTyrAlaGlnGlnThr			
GGCCGTATCAWCCTGTCCGGTRACACCGCTTACKCTCAGCAGAC			
CATAGTWGGACAGGCCAYTGTGGCGAATGMGAGTCGTCTGAGCT			

5

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SEQ ID NO: 14. It incorporates the asparagine mutation within the NS4a segment (numbered –1 in this fusion construct and numbered 30 in the NS4a sequence) and retains the alanine at NS3 position 7.

Figure 6 shows an alignment of the protein sequences of SEQ ID Nos: 1, 3, 12, 14, 16, 18, 20, 22 and 24. As seen in Figure 6, the linker segment of SEQ ID NO: 14 is two residues longer relative to the original linker used in SEQ ID Nos: 3 and 12.

The optimized turn sequence by itself is not sufficient to confer solubility on a NS4a-NS3 fusion. This can be seen in lanes 6 & 7 of Figure 4, where the optimized turn in combination with the wild-type α -helix 0 (SEQ ID NO: 24), does not confer high solubility. Only the presence of the α -helix0 mutations, either with or without the optimized turn sequence (see Figure 4 lanes 4 & 5 and lanes 8 & 9, respectively), allows high levels of fusion protease in the supernatant fractions.

However, the experiment shown in Figure 4 only shows whether the expressed protein fractionates in the soluble fraction. A more detailed analysis of the construct's suitability for structural studies was performed using NMR, where the protein aggregation state could be assessed. ^1H - ^{15}N HSQC NMR spectra were collected for ^{15}N -labeled proteins of SEQ ID NO: 12 and SEQ ID NO: 14. As shown in Figure 5, the protein of SEQ ID NO: 14 (panel B - mutant α -helix 0 with optimized linker) generates a higher quality HSQC spectrum (as judged by NMR linewidth and resolution of 2D peaks) than the protein produced from SEQ ID NO: 12 (panel A - mutant α -helix 0 with non-optimized linker). This analysis shows that the optimized linker derived in Experiment 5 is contributing favorably to the overall solubility and non-aggregation of the modified HCV NS4a-NS3 fusion protease.

Example 6 – Incorporation in modified HCV NS4a-NS3 fusion proteases of naturally-occurring hydrophobic-to-hydrophilic residue substitutions and change of non-zinc-binding cysteines to non-cysteine amino acids

Inspection of a protein sequence alignment of the HCV NS3 portions of different published HCV isolates (not shown) showed that many residue positions are variable among the different isolates. In particular, a number of solvent-exposed surface residue positions can take on a number of different naturally-occurring amino-

acid residue types that differ in their hydrophobic-hydrophilic character. These residues include Ala₃₉ (sometimes Ser), Ala₄₀ (sometimes Thr), Pro₆₇ (sometimes Ser), Ile₇₂ (sometimes Thr), and Pro₈₆ (sometimes Gln).

Substitution of non-essential cysteine residues is a method sometimes utilized in attempts to improve a protein's biochemical properties by reducing the tendency of the protein to form disulfide-linked multimers upon oxidation (for example, Yamazaki et al., *Protein Science* [1996] **5**, 495-506). Preliminary experiments showed that substitution of each of the four non-zinc-binding cysteines within HCV NS3 protease had minimal effects upon enzymatic activity (data not shown).

Inspection of the protein sequence alignment showed that, within the a given sequence, two of the non-zinc-binding cysteine residues (Cys₄₇ and Cys₅₂) either appeared together as a cysteine pair or were both changed to non-cysteine residue types (coupled with their spatial arrangement within the HCV NS3 protease sequence, this fact indicates that this pair is likely to form a disulfide linkage). A third non-zinc-binding cysteine residue (Cys₁₅₉), was also targeted for mutagenesis (although this position is invariant among the different HCV isolates inspected).

Using this information, six oligonucleotides were designed that code for alternate residue types at selected solvent-exposed residue positions, with a bias toward substitution of hydrophilic residue-types in place of hydrophobic ones. In addition, three of the four non-zinc-binding cysteine residues (Cys₄₇, Cys₅₂, and Cys₁₅₉) were changed to non-cysteine residues.

Surface Oligo#1 (P86Q):

5'-ACGGGAACCCTGCGGAGCTGCCAACCAACCAGGTCTTTG-3'

Surface Oligo#2 (P67P/S; I72Q):

5'-
CAACGTTGGTGTACATCTGGGTAACCGGACCTTTCGRGGAAGCGATGGTA
CGGGT-3'

Surface Oligo#3 (A39A/S; A40T):

5'-CCAGGAAGGTCTGGGTAGMGGTGGAAACGATCTGAAC-3'

Cys Oligo#1 (C159S/T);

5'-CTTTAGCAACACCACGGGTGGWAACAGCAGCACGGAAGAT-3'

5 Cys Oligo#2 (C47S/T; C52L/M);

5'-

ACCGTGGTAAACGGTCCACAKAACACCGTTGATGGWGGTAGCCAGGAAG
GTC-3'10 Cys Oligo#3 (A39A/S; A40T; C47S/T; C52L/M);

5'-

ACCGTGGTAAACGGTCCACAKAACACCGTTGATGGWGGTAGCCAGGAAG
GTCTGGGTAGMGGTGGAAACGATCTGAAC-3

15 Where W = (A,T); K = (G,T); M = (A,C); R = (A,G).

The site-directed mutations were introduced into SEQ ID NO:13 by the *dut-*
ung method (Kunkle, 1985) using the Muta-Gene Phagemid kit (BioRad). The
mutations were generated by using the six oligonucleotides in different combinations
to produce distinct sets of clones having different mutation combinations. Expression
levels and solubilities of the randomly-picked mutant NS4a-NS3 fusion protease
variants were monitored by SDS-PAGE analysis of the soluble fractions of induced
cell lysates (similar to the analysis shown in Figure 4) and some of the clones
exhibiting enhanced protease solubility were sequenced. The A40T, I72T, P86Q
mutations were found to have the most pronounced positive effect on solubility.

To combine these mutations with the optimized linker sequence generated in
Example 5, DNA sequences encoding the A40T, I72T, P86Q surface mutants were
subcloned into SEQ ID NO: 15 without and with the C47S, C52L, and C159S
cysteine mutants to produce the proteins presented in SEQ ID NO: 16 (Figure 14) and
SEQ ID NO: 18 (see Figure 15), respectively. The 2D ¹H-¹⁵N HSQC NMR spectrum
of protein produced from SEQ ID NO: 18 shown in Figure 5 panel C clearly indicates
that this protease variant is highly non-aggregating and suitable for high-resolution

NMR structural analysis. *E. coli* cells harboring the plasmid containing SEQ ID NO: 19, which is the DNA sequence encoding SEQ ID NO: 18, have been deposited with the ATCC and have ATCC accession number 207040.

Additional modified HCV NS4a-NS3 fusion proteases analogous to the one shown in SEQ ID NO: 18 were constructed in which another α -helix 0 variant sequence identified in Example 4 was substituted. Variant α -Helix 0 -7, identified as SEQ ID NO: 9 in Figure 11, was substituted, resulting in SEQ ID NO: 20 (see Figure 16) and SEQ ID NO: 22 (see Figure 17). SEQ ID NO: 22 is the same as SEQ ID NO: 20, except that an additional naturally-occurring amino-acid substitution (C16T) was included. When expressed, both of these modified HCV NS4a-NS3 fusion proteases had solubilities comparable to that of SEQ ID NO: 18 and, as expected, exhibited chromatographic properties on ion exchange media that differed somewhat from the SEQ ID NO: 18 homolog. 2D ^1H - ^{15}N HSQC spectra confirmed that these protein variants were similarly folded to that of the SEQ ID NO: 18 protease (data not shown). *E. coli* cells harboring the plasmid containing SEQ ID NO: 23, which is the DNA sequence encoding SEQ ID NO: 22, have been deposited with the ATCC and have ATCC accession number 207041.

Example 7 - Expression and Purification in *E. coli*

All constructs were expressed in *E. coli* strain BL21(DE3) (Novagen) using one of the pET plasmid vectors (Novogen). Proteins were expressed either as polyhistidine-tagged proteins using the pET28a vector, or as non-tagged proteins using the pET29a vector. Probably due to optimized bacterial codon usage and massive overproduction, expression of these constructs resulted in translational readthrough protein products (~10-20%), in addition to the predicted full-length protein product. Modification of the expression vectors to include a triple-stop set of codons (TAA TAA TGA) results in the elimination of the readthrough products (data not shown).

The following two purification methods are outlined for the modified HCV NS4a-NS3 fusion protease produced from expression of SEQ ID NO: 19 in the pET29a vector system (no tag). However, one skilled in the art could readily modify

the procedures slightly to purify any of the modified forms of HCV NS3 protease of the present invention.

Method 1

5 Expression of non-tagged variant expressed from SEQ ID NO: 19 was carried out in minimal bacterial growth media with induction with 0.3 mM IPTG when the cell density reached OD600 = 1.0. Concurrent with induction of the culture, ZnCl₂ was added to final 30 µM concentration and the cells were transferred to 20 degrees C for 20 hours.

10 After centrifugation, the cell pellet was resuspended in 25mM Na-phosphate buffer pH 7.5, 0.5M NaCl, 2mM DTT, 10 µM ZnCl₂ and cells were disrupted by passage through a High Pressure Homogenizer (RANNI model 8.30H). The homogenate was clarified at 15,000 (Sorvall model SS34 rotor) rpm for 30 min and 10 mM MgCl₂, and 20µg/ml DNase/RNase were added to the supernatant.

15 After incubation at room temperature for 10 minutes, the supernatant was diluted twice with 25mM Na-phosphate buffer, 2mM DTT, 10µM ZnCl₂ and applied onto Macro-Prep S column (Bio-Rad ,1kg of resin) equilibrated with 25mM Na-phosphate pH 7.5, 0.2M NaCl, 2mM DTT, 10µM ZnCl₂. After washing the column till OD280 ~ 0.1, the bound protein was eluted with the same buffer with 0.5 M NaCl.

20 The eluate was concentration on an Amicon YM5 membrane and applied onto a Superdex 30 26/60 column equilibrated with 25mM Na-phosphate pH-7.5, 0.2M NaCl, 2mM DTT, 10µM ZnCl₂. The fractions of the NS3 peak were applied onto SP Sepharose 26/10. Buffer A was the same buffer as for the Superdex 30 column. Buffer B is the same buffer with 1M NaCl. The protein peak elutes at 0.5-0.6 M NaCl.

25 For crystallization, the purified protein was exchanged into 0.5 M NaCl, 25 mM MES (2-(N-Morpholino)ethanesulfonic Acid), pH 6.5, 10% (v/v) glycerol, 2 mM dithiothreitol (DTT) and could be concentrated to 5 mM (~ 100 mg/ml). For NMR spectroscopy, the protein was exchanged into 25 mM sodium phosphate pH 6.5, 50 mM sodium sulfate, 2 mM deuterated DTT, and 10% D₂O and could be concentrated to at least 3 mM.

Integrity of the preparation was verified by mass spec analysis. Using this purification method, the final yields are typically 50-65 mg pure protein per liter culture.

Method 2

5 For protein for crystallography, the following modified protocol was found to produce a preparation that crystallized readily (overnight):

After cell disruption in the homogenizer (as in Method 1), the homogenate was centrifuged (Sorvall model SS34) for 30 min at 16,000 rpm. The supernatant was treated with PEI (polyethylenimine - 0.2% final) for 20 min at room temperature upon stirring. The white solution was centrifuged at 16,000rpm for 20min and supernatant was precipitated with ammonium sulfate (40%) at 4 degrees C for 30 min. The solution was centrifuged at 10,000 rpm for 30 min. The pellet was resuspended in 25mM Na-phosphate, pH 7.5, 2mM DTT, 10:M ZnCl₂ (10 ml per liter of culture) and centrifuged again at 16,000 rpm for 10 min. The supernatant was applied first onto Superdex 75 26/60 column equilibrated with 25mM Na -Phosphate buffer, pH 7.5, 0.2M NaCl, 2mM DTT, 10:M ZnCl₂. The peak fractions were applied then to an SP Sepharose 26/10 column. Buffers A and B are the same as in Method 1.

After concentration of peak functions on Amicon membrane, the protein was applied onto Superdex 30 16/60 equilibrated with 25mM Na-phosphate, pH 7.5, 2mM DTT, 10:M ZnCl₂ and no salt. Only the purest side fractions of HCV NS3 protease were collected and pooled.

After concentration (Millipore Ultrafree-5K cutoff), the protein was exchanged into 25mM MES buffer, pH 6.5, 0.5M NaCl, 2mM DTT, 10:M ZnCl₂. The protein preparation concentrated easily and readily produced crystals (as outlined in Example 10) even after four months of storage at 4 degrees C.

Example 8 – NMR spectroscopy of modified HCV NS4a-NS3 fusion proteases

Modified HCV NS4a-NS3 fusion proteases were prepared for NMR analysis by exchanging the purified protein (see Method 1, Example 7) into NMR buffer (25 mM sodium phosphate pH 6.5, 50 mM sodium sulfate, 2 mM deuterated DTT, and 10% D₂O). Protease samples both with and without readthrough product (see

Example 7) were successfully used for NMR spectroscopy in both Examples 8 and 9. Sample concentrations ranged from 0.2 mM to 3 mM.

Two-dimensional ^1H - ^{15}N HSQC NMR spectra were obtained using a WATERGATE HSQC pulse sequence (Mori et al., (1995), *J. Magn. Reson.* **B108**, 94 - 98; Sklenar, (1995) *J. Magn. Res.* **A114**, 132-135) on a Varian UNITY PLUS 600 MHz NMR spectrometer. The data were collected at 30 degrees C with 4 transients per FID and either 128 or 256 increments, with spectral widths of 10.0 and 2.4 kHz in F_2 (^1H) and F_1 (^{15}N), respectively.

A 1.5 mM solutions of a double-labeled (^{13}C - ^{15}N) preparation of apo-HCV protease (SEQ ID NO:18) was prepared. A full set of NMR spectra were collected and used to determine the backbone NMR resonances of the apo-HCV protease. The 3D NMR experiments included HNC0, HNCAC0, HNCACB, CBCACONH, HBHACONH, HNCAHA, HCCH-TOCSY, ^{15}N -edited NOESY and ^{13}C -edited NOESY (see Clore and Gronenborn, *Meth. Enzymol.* **239**, 349-363 (1994) for references to these experiments).

Backbone NMR resonances for 155 of the 187 non-proline residues and 8 of the 11 proline residues were obtained along with most of the sidechain assignments. These assignments include the catalytic triad residues His₅₇, Asp₈₁, and Ser₁₃₉ and the residues spatially near to them, indicating that the apo-form of the protein is a good reagent for NMR analysis of protease:inhibitor complexes

Example 9 – NMR of a complex of a modified HCV NS4a-NS3 fusion protease with an inhibitor

A complex between a ^{15}N -labeled HCV NS4a-NS3 fusion protease (SEQ ID NO: 18) and an inhibitor peptide (Ac-Asp-D-Glu-Leu-Ile-Cha-Cys-OH) (Ingallinella et al., *Biochemistry* **37**, 8906-8914 (1998)) was formed by forming a 1:1 complex of the two components (at 200 μM concentration) in the NMR buffer described in Example 8. A 2D HSQC spectrum was collected. The HSQC spectra of the apo- and peptide-complexed soluble modified HCV NS4a-NS3 fusion protease (SEQ ID NO: 18) are overlaid in Figure 7. Many residues undergo chemical shift perturbations upon addition of the inhibitor, including the active site residues.

Example 10 – Crystal Structure of a complex of modified HCV NS4a-NS3 fusion protease with an inhibitor

The modified HCV NS4a-NS3 fusion protease produced by Method 2 in
 5 Example 7 is well suited to support X-ray crystallographic studies. In this Example, the protein preparations included 10-20% translational readthrough product (see Example 7). The preparations produced crystals of a complex with inhibitor overnight and a structure of the complex with inhibitor to 2.1 Å resolution.

Crystals of the soluble modified HCV NS4a-NS3 fusion protease complexed
 10 with peptidic inhibitor Ac-Asp-D-Glu-Leu-Ile-Cha-Cys-OH [$IC_{50} = 15$ nM, Ingallinella et al., *Biochemistry* **37**, 8906-8914 (1998)] were grown by standard hanging-drop vapor-diffusion methods at room temperature. Protein solution: 21.6 mg/ml protein in 0.5 M NaCl, 25 mM MES, pH 6.5, 10% (v/v) glycerol, 2 mM DTT, and 5.64 mM inhibitor (6X molar excess) incubated at room temperature for 2 hours.
 15 Reservoir solution: 2 M ammonium sulfate, 0.1 M sodium acetate, pH 4.6, 1% (v/v) PEG monomethyl ester 350, 5 mM zinc chloride. Droplets were composed of equal-volume aliquots of protein and reservoir solutions. Crystals were obtained overnight by these conditions.

A crystal was taken from its droplet and placed in a small volume of reservoir
 20 solution that had been made 20% (v/v) in glycerol. It was extracted with a standard Hampton fiber loop mounted in a Hampton pin and immediately introduced into the 100K nitrogen stream from an Oxford Cryosystems low-temperature device.

Data to 2 Å resolution were collected from this crystal on a rotating-anode source (CuK α) with an R-Axis II detector. Completeness is 94% from 20-2 Å resolution,
 25 and 64% in the outer shell (2.07-2.00 Å), with R(sym)s of 9.1% and 39.7% for all and for outer shell, respectively.

X-ray diffraction from this crystal indicates space group $P4_12_12$ with unit cell parameters $a = b = 67.1$, $c = 81.2$ Å and one molecule per asymmetric unit.

The structure was solved by application of standard molecular-replacement
 30 techniques with a NS4a-NS3 fusion search model based upon a previously reported structure of HCV protease:NS4a complex whose coordinates are on deposit with the Protein Data Bank [1JXP.pdb as described in *Protein Sci.* **7**, 837-847 (1998)]. Refinement of the structure by XPLOR with data from 20.0-2.1 Å resolution and

F/(F)>1.0 was suspended at an R-factor of 19.7% and R-free of 27.6%. The current model (1) includes about 120 water molecules, of which about 2/3 were added by an automated routine and have not been checked in the electron density map, (2) lacks modeled residues for loops at 0-4 and 87-89 for which density is not clear, and (3) includes no alternate conformations for side chains although several were found.

Electron density for the entire inhibitor is clearly seen, and its conformation and that of its binding site on the protein are unambiguously defined, thus making the structure immediately useful for drug design purposes. The quality of the inhibitor map can be seen in Figure 8. The inhibitor binds entirely on the P side of the active site and the carboxylate at its C-terminus binds in a pocket that corresponds to the oxyanion hole of the classical serine protease active site. Away from the active site, a zinc ion is coordinated by the side chains of cysteines at 97, 99, and 145, and by a water molecule, although the identity of this latter ligand is questioned. As the B factor of this water refines to 2 Å² and there is a large residual peak in the difference map at its position, it is suspected of being another component of the crystallization fluid and we have refined it as a chloride ion.

The above examples are illustrative and do not limit the claims.

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